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Enclosed is a copy of Priority Document EP 99202068.5 filed June 25, 1999 for the above referenced application.

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Der Präsident des Europäischen Patentamts;
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Le Président de l'Office européen des brevets
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Nucleic acid binding of multi-zinc finger transcription factors

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Nucleic acid binding of multi-zinc finger transcription factors

Field of the invention

The invention concerns a method of identifying transcription factors such as activators and/or repressors comprising providing cells with a nucleic acid sequence at least comprising a sequence CACCT as bait for the screening of a library encoding potential transcription factors and performing a specificity test to isolate said factors. Preferably the bait comprises twice the CACCT sequence, more particularly the bait comprises one of the sequences CACCT-N-CACCT, CACCT-N-AGGTG, AGGTG-N-CACCT or AGGTG-N-AGGTG wherein N is a spacer sequence of at least 8 base pairs.

The identified transcription factor(s) using the method according to the invention comprises separated clusters of zinc fingers such as for example a two-handed zinc finger transcription factor.

Background of the invention

Zinc fingers are among the most common DNA binding motifs found in eukaryotes. It is estimated that there are 500 zinc finger proteins encoded by the yeast genome and that perhaps 1 % of all mammalian genes encode zinc finger containing proteins. These are classified according to the number and position of the cysteine and histidine residues available for zinc coordination. The CCHH class, which is typified by the *Xenopus* transcription factor IIIA (19), is the largest. These proteins contain two or more fingers in tandem repeats. In contrast, the steroid receptors contain only cysteine residues that form two types of zinc-coordinated structures with four (C_4) and five (C_5) cysteines (28). The third class of zinc fingers contains the CCHC fingers. The CCHC fingers which are found in *Drosophila*, and in mammalian and retroviral proteins, display the consensus sequence C-X_2-C-X_4-H-X_4-C (7, 21, 24). Recently, a novel configuration of CCHC finger, of the C-X_5-C-X_{12}-H-X_4-C type, was found in the neural zinc finger factor/myelin transcription factor family (11, 12, 36). Finally, several yeast transcription factors such as GAL4 and CHA4 contain an

atypical C_6 zinc finger structure that coordinates 2 zinc ions (9, 32).

Zinc fingers are usually found in multiple copies (up to 37) per protein. These copies can be organized in tandem array, forming a single cluster or multiple clusters, or they can be dispersed throughout the protein. Several families of transcription factors share the same overall structure by having two (or three) widely separated clusters of zinc fingers in their protein sequence. The first, the MBPs/PRDII-BF1 transcription factor family, includes *Drosophila Schnurri* and *Spalt* genes (1, 3, 6, 14, 33). Both MBP-1 (also known as PRDII-BF1) and MBP-2 contain two widely separated clusters of two CCHH zinc fingers. The overall similarity between MBP-1 and MBP-2 is 51%, but the conservation is much higher (over 90%) for both the N-terminal and the C-terminal zinc finger clusters (33). This indicates an important role of both clusters in the function of these proteins. In addition, the N-terminal and C-terminal zinc finger clusters of MBP-1 are very homologous to each other (3).

The neural specific zinc finger factor 1 and factor 3 (NZF-1 and NZF-3), as well as the myelin transcription factor 1 (MyT1 , also known as NZF-2), belong to another family of proteins containing two widely separated clusters of CCHC zinc fingers (11, 12, 36). Like the MBP proteins, different NZF factors exhibit a high degree of sequence identity (over 80%) between the respective zinc finger clusters, whereas the sequences outside of the zinc finger region are largely divergent (36). In addition, each of these clusters can independently bind to DNA, and recognizes similar core consensus sequences (11). NZF-3 binds to a DNA element containing a single copy of this consensus sequence but was shown to exibit a marked enhancement in relative affinity to a bipartite element containing two copies of this sequence (36). This suggests that the NZF factors may also bind to reitirated sequences. However, the mechanism underlying the cooperative binding of NZF-3 to the bipartite element is currently unknown.

The *Drosophila Zfh-1* and the vertebrate δEF1 proteins (also known as ZEB or AREB6) belong to a third family of transcription factors. This family is characterized by the presence of two separated clusters of CCHH zinc fingers and a homeodomain-like structure (see Fig. 1A)(4, 5, 35). In δEF1, the N-terminal and C-terminal clusters are also very homologous

and were shown to bind independently to very similar core consensus sequences (10). Recently, it was shown that mutant forms of δEF1 lacking either the N-terminal or the C-terminal cluster have lost their DNA binding capacity indicating that both cluster are required for the binding of δEF1 to DNA (31).

Finally, the Evi-1 transcription factor was shown to contain 10 CCHH zinc fingers; seven zinc fingers are present in the N-terminal region, and three zinc fingers are in the C-terminal region (22). With this factor the situation is different from the transcription factors described above, because the two clusters bind to two different target sequences, which are bound simultaneously by full-length Evi-1 (20). Binding of full-length Evi-1 is mainly observed when the two target sequences are positioned in a certain relative orientation, but there was no strict requirement for an optimal spacing between these two targets.

Summary of the invention

The mechanism of DNA binding remains poorly understood for most of the above mentioned complex factors. It is our invention to characterise the DNA binding properties of vertebrate transcription factors belonging to the emerging family of two-handed zinc finger transcription factors like δEF1 and SIP1. SIP1 is a member of this transcription factor family, which was recently isolated and characterized as a Smad-interacting protein (34). Said SIP1 and δEF1, a transcriptional repressor involved in skeletal development and muscle cell differentiation, belong to the same family of transcription factors. They contain two separated clusters of CCHH zinc fingers, which share high sequence identity (>90%).

The DNA-binding properties of these transcription factors have been investigated. The N-terminal and C-terminal clusters of SIP1 show high sequence homology as well, and according to the invention each binds to a 5'-CACCT sequence. Furthermore, high affinity binding sites for full length SIP1 and δEF1 in the promoter regions of candidate target genes like Brachyury, α4-integrin and E-cadherin, are bipartite elements composed of one CACCT sequence and one CACCTG sequence. No strict requirement for the relative orientation of both sequences was observed, and the spacing between them may vary from

8 to at least 44 bp. For binding to these bipartite elements, the integrity of both SIP1 zinc finger clusters is necessary, indicating that they are both involved in binding to DNA. Futhermore SIP1 binds as a monomer to a CACCT-X_N-CACCTG site, by having one zinc finger cluster contacting the CACCT, and the other zinc finger cluster binding to the CACCTG sequence. This novel mode of binding may be generalised to other transcription factors that contain separated clusters of zinc fingers and may be applied to other Smad-binding proteins.

The invention thus concerns a method of identifying transcription factors such as activators and/or repressors comprising providing cells with a nucleic acid sequence at least comprising a sequence CACCT, preferably twice the CACCT sequence as bait for the screening of a library encoding potential transcription factors and performing a specificity test to isolate said factors. In another embodiment the bait comprises one of the sequences CACCT-N-CACCT, CACCT-N-AGGTG, AGGTG-N-CACCT or AGGTG-N-AGGTG wherein N is a spacer sequence of at least 8 base pairs.

The identified transcription factor(s) using the method according to the invention comprises separated clusters of zinc fingers such as for example a two-handed zinc finger transcription factor.

The sequence above mentioned may originate from any promoter region but preferably of the group selected from Brachyury, α4-integrin, follistatin or E-cadherin.

Part of the invention are the transcription factors obtainable by above referenced method as well.

In another embodiment the present invention relates to a method of identifying compounds with an interference capability towards transcription factors, obtained as above mentioned, by

a) adding a sample comprising a potential compound to be identified to a test system composed of (i) an oligo nucleotide sequence comprising one of the sequences CACCT-N-CACCT, CACCT-N-AGGTG, AGGTG-N-CACCT or AGGTG-N-AGGTG as bait wherein N is a spacer sequence of at least 8 base pairs, (ii) a protein capable to

bind said oligonucleotide sequence,

b) incubating said sample in said system for a period sufficient to permit interaction of the compound or its derivative or counterpart thereof with said protein and

c) comparing the amount and/or activity of the protein bound to the oligo nucleotide sequence before and after said adding.

Comparison of the amount of protein bound to the oligo nucleotide sequence before and after adding the test sample can be accomplished, for example, using a gel band-shift assay or a filter binding assay.

As a next step the compound thus identified can be isolated and optionally purified and further analysed according to methods known to persons skilled in the art.

To the scope of the present invention also belongs a test kit to perform said method comprising at least (i) an oligo nucleotide sequence comprising one of the sequences CACCT-N-CACCT, CACCT-N-AGGTG, AGGTG-N-CACCT or AGGTG-N-AGGTG wherein N is a spacer sequence of at least 8 base pairs and (ii) a protein capable to bind said oligonucleotide sequence. This protein may be for instance a Smad protein.

In another embodiment the current invention concerns an alternative to the so-called two hybrid screening assay as disclosed in the prior art. Several means and methods have been developed to identify binding partners of transcription factors. This has resulted in the identification of a number of respective binding proteins. Many of these proteins have been found using so-called two hybrid systems. Two-hybrid cloning systems have been developed in several labs (Chien et al., 1991; Durfee et al., 1993; Gyuris et al., 1993). All have three basic components: Yeast vectors for expression of a known protein fused to a DNA-binding domain, yeast vectors that direct expression of cDNA-encoded proteins fused to a transcription activation domain, and yeast reporter genes that contain binding sites for the DNA-binding domain. These components differ in detail from one system to the other. All systems utilise the DNA binding domain from either Gal4 or LexA. The Gal4 domain is efficiently localised to the yeast nucleus where it binds with high affinity to well-defined binding sites which can be placed upstream of reporter genes (Silver et al., 1986). LexA

does not have a nuclear localisation signal, but enters the yeast nucleus and, when expressed at a sufficient level, efficiently occupies LexA binding sites (operators) placed upstream of a reporter gene (Brent et al., 1985). No endogenous yeast proteins bind to the LexA operators. Different systems also utilise different reporters. Most systems use a reporter that has a yeast promoter, either from the GAL1 gene or the CYC1 gene, fused to lacZ (Yocum et al., 1984). These lacZ fusions either reside on multicopy yeast plasmids or are integrated into a yeast chromosome. To make the lacZ fusions into appropriate reporters, the GAL1 or CYC1 transcription regulatory regions have been removed and replaced with binding sites that are recognised by the DNA-binding domain being used. A screen for activation of the lacZ reporters is performed by plating yeast on indicator plates that contain X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside); on this medium yeast in which the reporters are transcribed produce beta-galactosidase and turn blue. Some systems use a second reporter gene and a yeast strain that requires expression of this reporter to grow on a particular medium. These "selectable marker" genes usually encode enzymes required for the biosynthesis of an amino acid. Such reporters have the marked advantage of providing a selection for cDNAs that encode interacting proteins, rather than a visual screen for blue yeast. To make appropriate reporters from the marker genes their upstream transcription regulatory elements have been replaced by binding sites for a DNA-binding domain. The HIS3 and LEU2 genes have both been used as reporters in conjunction with appropriate yeast strains that require their expression to grow on media lacking either histidine or leucine, respectively. Finally, different systems use different means to express activation-tagged cDNA proteins. In all current schemes the cDNA-encoded proteins are expressed with an activation domain at the amino terminus. The activation domains used include the strong activation domain from Gal4, the very strong activation domain from the Herpes simplex virus protein VP16, or a weaker activation domain derived from bacteria, called B42. The activation-tagged cDNA-encoded proteins are expressed either from a constitutive promoter, or from a conditional promoter such as that of the GAL1 gene. Use of a conditional promoter makes it possible to quickly demonstrate that activation of the reporter gene is dependent on expression of the

activation-tagged cDNA proteins.

It is clear from the discussion above that two-hybrid systems for finding binding proteins have been used in the past.

However, although the conventional two hybrid system has proven to be a valuable tool in finding proteinaceous molecules that can bind to other proteins it is a (very) artificial system. A characteristic of any two hybrid system is that a fusion protein is made consisting of a part of which binding partners are sought and a reporter part that enables detection of binding. For finding relevant binding partners several criteria must be met of which one is of course the correct choice of the region in said protein where binding to other proteins occurs. Another criterion which is much more difficult if not impossible to predict accurately on forehand is obtaining correct folding of said region (i.e. a folding of said region sufficiently similar to the folding of said region in the natural protein). Correct folding depends among others on the actual amino-acid sequence chosen for generating said fusion protein. Another factor determining the identification of relevant binding partners is the sensitivity with which binding can be detected.

An alternative to the above mentioned conventional two hybrid system is herewith provided in the current invention. Thus an alternative object of the invention is to provide an in vivo method and a kit for detecting interactions between proteins and the influence of other compounds on said interaction as such, using reconstitution of the activity of a transcriptional activator. This reconstitution makes use of two, so-called hybrid, chimeric or fused proteins. These two fused proteins each show, independently from one another, a weak affinity towards a nucleic acid sequence comprising one of the sequences CACCT-N-CACCT, CACCT-N-AGGTG, AGGTG-N-CACCT or AGGTG-N-AGGTG wherein N is a spacer sequence of at least 8 base pairs. However when both fused proteins are independently being bound to said sequence and the test proteins each available in each of two fused proteins are as a result thereof brought into close proximity, the binding affinity towards said nucleic acid sequence comprising one of the sequences CACCT-N-CACCT, CACCT-N-AGGTG, AGGTG-N-CACCT or AGGTG-N-AGGTG wherein N is a spacer

sequence of at least 8 base pairs becomes much stronger.

If the two test proteins indeed are able to interact, they bring as a consequence thereof into close proximity the two domains of the transcriptional activator. This proximity is sufficient to cause transcription, which can be detected by the activity of a marker gene located adjacent to the nucleic acid sequence comprising one of the sequences CACCT-N-CACCT, CACCT-N-AGGTG, AGGTG-N-CACCT or AGGTG-N-AGGTG wherein N is a spacer sequence of at least 8 base pairs.

In accordance herewith a method is provided for detecting an interaction between a first interacting protein and a second interacting protein comprising

a) providing a suitable host cell with a first fusion protein comprising a first interacting protein fused to a DNA binding domain capable to bind a nucleic acid sequence comprising one of the sequences CACCT-N-CACCT, CACCT-N-AGGTG, AGGTG-N-CACCT or AGGTG-N-AGGTG wherein N is a spacer sequence of at least 8 base pairs,

b) providing said suitable host cell with a second fusion protein comprising a second interacting protein fused to a DNA binding domain capable to bind a nucleic acid sequence comprising one of the sequences CACCT-N-CACCT, CACCT-N-AGGTG, AGGTG-N-CACCT or AGGTG-N-AGGTG wherein N is a spacer sequence of at least 8 base pairs,

c) subjecting said host cell to conditions under which the first interacting protein and the second interacting protein are brought into close proximity and

d) determining whether a detectable gene present in the host cell and located adjacent to said nucleic acid sequence has been expressed to a degree greater than expressed in the absence of the interaction between the first and the second interacting protein.

Definitons

The following definitions are set forth to illustrate and define the meaning and scope of the various terms used to describe the invention herein and their meaning is further elaborated hereunder for sake of clarity.

"Nucleic acid" or "nucleic acid sequence" or "nucleotide sequence" means genomic DNA, cDNA, double stranded or single stranded DNA, messenger RNA or any form of nucleic acid sequence known to a skilled person.

The terms "protein" and "polypeptide" used in this application are interchangeable. "Polypeptide" refers to a polymer of amino acids (amino acid sequence) and does not refer to a specific length of the molecule. Thus peptides and oligopeptides are included within the definition of polypeptide. This term does also refer to or include post-translational modifications of the polypeptide, for example, glycosylations, acetylations, phosphorylations and the like. Included within the definition are, for example, polypeptides containing one or more analogs of an amino acid (including, for example, unnatural amino acids, etc.), polypeptides with substituted linkages, as well as other modifications known in the art, both naturally occurring and non-naturally occurring.

The proteins and polypeptides described above are not necessarily translated from a designated nucleic acid sequence; the polypeptides may be generated in any manner, including for example, chemical synthesis, or expression of a recombinant expression system, or isolation from a suitable viral system. The polypeptides may include one or more analogs of amino acids, phosphorylated amino acids or unnatural amino acids. Methods of inserting analogs of amino acids into a sequence are known in the art. The polypeptides may also include one or more labels, which are known to those skilled in the art. In this context, it is also understood that the proteins may be further modified by conventional methods known in the art. By providing the proteins it is also possible to determine fragments which retain biological activity, namely the mature, processed form. This allows the construction of chimeric proteins and peptides comprising an amino sequence derived

from the mature protein which is crucial for its binding activity. The other functional amino acid sequences may be either physically linked by, e.g., chemical means to the proteins or may be fused by recombinant DNA techniques well known in the art.

The term "derivative", "functional fragment of a sequence" or " functional part of a sequence" means a truncated sequence of the original sequence referred to. The truncated sequence (nucleic acid or protein sequence) can vary widely in length; the minimum size being a sequence of sufficient size to provide a sequence with at least a comparable function and/or activity of the original sequence referred to, while the maximum size is not critical. In some applications, the maximum size usually is not substantially greater than that required to provide the desired activity and/or function(s) of the original sequence. Typically, the truncated amino acid sequence will range from about 5 to about 60 amino acids in length. More typically, however, the sequence will be a maximum of about 50 amino acids in length, preferably a maximum of about 30 amino acids. It is usually desirable to select sequences of at least about 10, 12 or 15 amino acids, up to a maximum of about 20 or 25 amino acids.

The terms "gene(s)", "polynucleotide", "nucleic acid sequence", "nucleotide sequence", "DNA sequence" or "nucleic acid molecule(s)" as used herein refers to a polymeric form of nucleotides of any length, either ribonucleotides or deoxyribonucleotides. This term refers only to the primary structure of the molecule. Thus, this term includes double- and single-stranded DNA, and RNA. It also includes known types of modifications, for example, methylation, "caps" substitution of one or more of the naturally occuring nucleotides with an analog.

A "coding sequence" is a nucleotide sequence which is transcribed into mRNA and/or translated into a polypeptide when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a translation start codon at the 5'-terminus and a translation stop codon at the 3'-terminus. A coding

sequence can include, but is not limited to mRNA, cDNA, recombinant nucleotide sequences or genomic DNA, while introns may be present as well under certain circumstances.

With "transcription factor" is meant a class of proteins that bind to a promoter or to a nearby sequence of DNA to facilitate or prevent transcription initiation.

With "promoter" is meant an oriented DNA sequence recognized by the RNA polymerase holoenzyme to initiate transcription.

With "RNA polymerase" is meant a multisubunit enzyme that synthesizes RNA complementary to the DNA template.

With "holoenzyme" is meant an active form of enzyme that consists of multiple subunits.

Detailed description of the invention

SIP1 and δEF1 bind to target sites containing one CACCT sequence and one CACCTG sequence

The DNA binding properties of SIP1 were studied. SIP1, a recently isolated Smad-interacting protein, belongs to the emerging family of two-handed zinc finger transcription factors (34). The organization of SIP1 is very similar to that of δEF1, the prototype member of this family. Both proteins contain two widely separated clusters of zinc fingers, which are involved in binding to DNA. The amino acid sequence homology is very high (more than 90%) within these two zinc finger clusters, whereas it is less evident in the other regions. This finding suggests that both proteins would bind in an analogous fashion to similar DNA targets. Indeed SIP1 as well as δEF1 bind with comparable affinities to many different target sites, which always contain two CACCT sequences. For all the target sites tested

here, the integrity of both CACCT sequences is absolutely necessary for the binding of either SIP1 or δEF1.

$SIP1_{FS}$ inhibits *Xbra2* expression when overexpressed in the *Xenopus* embryo (34), and $SIP1_{FS}$ binds to the *Xbra2* promoter by contacting two CACCT sequences. Recent studies using *Xenopus* transgenic embryos have shown that 2.1 kb of *Xbra2* promoter sequences suffice to express a reporter protein in the same domain as *Xbra* itself (17). However, a single point mutation within the downstream CACCT site (Xbra-D) in the promoter that disrupts SIP1 binding (as seen in gel retardation assays) has a severe effect. Expression of the marker protein initiates earlier (i.e. at stage 9), and is now found at ectopic sites, e.g. in the majority of ectodermal, mesodermal and endodermal cells (17). This indicates that this nucleotide, which is located within the downstream CACCT site, is required for correct spatial and temporal expression of the *Xbra2* gene. In addition, when a mutation is introduced in the upstream CACCT sequence, we observed the same premature and ectopic expression of *Xbra2* as for the mutation within the downstream CACCT site. Therefore, mutations in either the downstream or upstream CACCT that are known to affect SIP1 or δEF1 binding in EMSA, give the same phenotype *in vivo*, indicating that a *Xenopus* δEF1-like protein participates in the regulation of the *Xbra2* gene. In addition, these *in vivo* data support the conclusions from the *in vitro* binding experiments presented here : SIP1/δEF1-like transcription factors require two CACCT sites for regulating the expression of the *Xbra2* promoter.

Not all promoter regions containing two CACCT sequences represent SIP1 or δEF1 binding sites. Notably, duplication of the Xbra-F probe, which contains the upstream CACCT sequence present in the Xbra-WT element, is refractory to binding of either SIP1 or δEF1. Moreover, neither $SIP1_{NZF}$ nor $SIP1_{CZF}$ can bind efficiently to this site (Xbra-F) as monomer or as dimer. This strongly suggests that other sequences in addition to CACCT may be required for generating a high-affinity binding site. It appears that CACCTG is always a better target site for binding of these zinc finger clusters. Indeed, the high-affinity CACCTG site (Xbra-E) was shown to bind either the $SIP1_{NZF}$ or the $SIP1_{CZF}$ cluster. In addition,

modification of the CACCTG site into CACCTA strongly affects the binding of $SIP1_{FS}$ and δEF1 to the Xbra promoter, confirming the importance of this 3'-guanine residue. By comparing the sequence of all the SIP1 and δEF1 target sites, a minimal consensus sequence was found composed of one CACCT sequence and one CACCTG sequence, demonstrating that these two sequences are sufficient to form a high-affinity binding site for SIP1 or δEF1.

Although the upstream CACCT sequence is unable to bind $SIP1_{CZF}$ or $SIP1_{NZF}$, this sequence is contacted by full size SIP1 in the context of the Xbra-WT probe. The upstream CACCT sequence is a prerequisite for the binding of $SIP1_{FS}$ to the Xbra-WT probe. Thus, when the upstream CACCT sequence is combined with another, high-affinity CACCTG site (Xbra-E), this low affinity site (Xbra-F) becomes committed to the binding of $SIP1_{FS}$. A model in which $SIP1_{FS}$ contacts its target promoter via the binding of one of its zinc fingers clusters to a high affinity CACCTG-sequence (e.g. Xbra-E) is favoured, which is followed by the contact of the low affinity CACCT site (Xbra-F) by the second cluster, and this additional interaction strongly stabilizes SIP1 binding. Therefore, a CACCT site may still have an important function in the regulation of gene expression, while even on its own it neither binds $SIP1_{NZF}$, $SIP1_{CZF}$ nor $SIP1_{FS}$.

The DC5 probe from the δ1-crystallin enhancer was previously shown to bind specifically δEF1 (31). However, this probe contains only one CACCT sequence. Therefore, despite having demonstrated here that high affinity binding sites for δEF1 should contain one CACCT sequence and one CACCTG sequence, it cannot be excluded that in particular cases, such as the DC5 probe, one CACCT site would be sufficient for the binding of this type of transcription factor.

Mode of SIP1 DNA binding

When tested independently in EMSA, both the C-terminal as well as the N-terminal zinc finger clusters of SIP1 or δEF1 bind to very similar CACCT-containing consensus sequences. Both for SIP1 and δEF1, NZF3 and NZF4 share an extensive amino acid

sequence homology with CZF2 and CZF3, respectively. This homology may explain why these two clusters can bind to similar consensus sequences. In addition, it has been shown that SIP1 or δEF1 require two CACCT sequences for binding to several potential target sites. Based on these results, it is proposed that SIP1 and δEF1 would bind to their target elements in such a way that one zinc finger cluster contacts one of the CACCT sites, while the other cluster contacts the second CACCT site (see figure 8, model 1). An alternative model would be that SIP1 or δEF1 homodimerizes before being able to bind to these target sites with high affinity (model 2). The DNA binding capacity of $SIP1_{NZF}$ is abolished by mutations in either NZF3 or NZF4. Similarly, mutations within CZF2 or CZF3 also affect the binding capacity of $SIP1_{CZF}$. When these mutations are introduced in the context of the full size SIP1, binding of $SIP1_{FS}$ is not observed any longer. This clearly indicates that the binding activity of both zinc finger clusters is required for the binding of $SIP1_{FS}$ to its target element, containing a doublet of CACCT sites. Similarly, it was previously shown that the integrity of both zinc finger clusters of δEF1 is also necessary for binding DNA (31). These observations indicate that both zinc fingers clusters are contacting directly the DNA. Therefore, in the dimer model (Fig. 8, model 2), the $SIP1_{NZF}$ of one SIP1 molecule should bind to one CACCT sequence and the $SIP1_{CZF}$ of the second SIP1 molecule should contact the other CACCT sequence. If such a dimer configuration would exist, then it can be assumed that certain combinations of full size SIP1 molecules having different mutations within CZF or NZF, respectively, should allow the formation of functional dimer which is able to bind to its target DNA. None of the possible combinations of the four $SIP1_{FS}$ mutants tested (NZF3mut, NZF4mut, CZF2mut and CZF3mut) gave rise to a DNA/SIP1 complex in EMSAs. This argues against the existence of SIP1 dimers. In addition, using differently tagged $SIP1_{FS}$ molecules, detection of SIP1 dimers in EMSAs was not possible, nor to supershift such dimeric complexes with different antibodies. Therefore support is provided to model 1 in which SIP1 binds as a monomer to a target site, which contains one CACCT sequence and one CACCTG sequence.

It has been shown in this invention that neither the relative orientation of the two CACCT

sequences nor the spacing between these sequences is critical for the binding of $SIP1_{FS}$ or δEF1. This demonstrates that these transcription factors should display a highly flexible secondary structure to accommodate the binding to these different target sites. The long linker region between the two zinc finger clusters within SIP1 and δEF1 may permit this flexibility in the secondary structure of these proteins. These transcription factors can bind to sites containing CACCT sequences separated by at least 44 bp (Ecad-WT), suggesting that a region of about 50 bp of promoter sequences might be covered and therefore less accessible to transcriptional activators once $SIP1_{FS}$ or δEF1 is bound to this promoter. This indicates that SIP1 or δEF1 could function as transcriptional repressor by competing with transcriptional activators that bind in this region covered by SIP1 or δEF1.

Other families of transcription factors may bind DNA with a similar mechanism as SIP1

This new mode of DNA binding may also be generalized to other transcription factor families, which, like SIP1 and δEF1, contain separated clusters of zinc fingers like those of the MBP/PRDII-BF1 family (1, 3, 6, 29, 33). Like for SIP1 and δEF1, the conservation of these zinc finger clusters is very strong between the different members of this family (1). In addition, the C-terminal cluster is very homologous to the N-terminal cluster and, in the case of PRDII-BF1, these clusters bind to the same sequences when tested independently (3). Therefore, this type of transcription factor may bind to two reiterated sequences through the contact of one zinc finger cluster with one sequence and the other cluster with the second sequence. Similarly, the different members of the NZF family of transcription factors also have two widely separated clusters of zinc fingers (11, 12, 36). MyT1, NZF-1 and NZF-3 all bind to the same consensus element AAAGTTT. Like for SIP1 and δEF1, which show a significantly higher affinity to elements containing 2 CACCT sequences, an element containing 2 AAAGTTT sequences demonstrated a markedly higher affinity to NZF-3 (36). This suggests that 2 AAAGTTT sequences are also necessary to create a high-affinity binding site for these transcription factors, and that they may bind DNA with a similar

mechanism as SIP1 and δEF1. Finally, the Evi-1 protein, which contains 7 zinc fingers at the N-terminus and 3 zinc fingers at the C-terminus, binds to two consensus sequences. It binds to a complex consensus sequence (GACAAGATAAGATAA-N_{1-28}-CTCATCTTC) via a mechanism that may involve the binding of the N-terminal zinc finger cluster to the first part and the binding of the C-terminal cluster to the second part (20). In conclusion, the mode of DNA-binding that is described here may not only be applicable to the SIP1/δEF1 family of transcription factors, but is more universal.

SIP1 was cloned as a Smad1-interacting protein but was also shown to interact with Smad2, 3 and 5 (34). Smad proteins are signal transducers involved in the BMP/TGF-β signaling cascade (13). Upon binding of TGF-β ligands to the serine/threonine kinase receptor complex, the receptor-regulated Smad proteins are phosphorylated by type I receptors and migrate to the nucleus where they modulate transcription of target genes. The interaction between SIP1 and Smads is only observed upon ligand stimulation, indicating that Smads need to be activated before they are capable of interacting with SIP1 (34). Surprisingly, Evi-1, a transcription factor that may bind DNA with a similar mechanism as SIP1, is a Smad3-interacting protein (15). So far, it was shown that Evi-1 inhibited the binding of Smad3 to DNA but certainly has an effect on target promoters of Evi-1. *Schnurri*, which is the *Drosophila* homologue of the human PRDII-BF1 transcription factor, is a protein that may also bind DNA with a similar mechanism as SIP1 protein. Interestingly, *Schnurri* was proposed to be a nuclear protein target in the dpp-signaling pathway (1, 6). Dpp is a member of the TGF-β family. This makes *Schnurri* a candidate nuclear target for *Drosophila* Mad protein, the *Drosophila* homologue of vertebrate Smads. Therefore it is postulated that the mode of DNA binding employed by SIP1 can be generalized to other zinc finger containing Smad-interacting proteins, and may represent a common feature of several Smad partners in the nucleus.

Based on these results, a novel mode of DNA binding for δEF1 family of transcription factors is demonstrated. This mode of DNA binding may also be relevant to other families of transcription factor that contains separated clusters of zinc fingers.

Materials and methods used in this invention

Plasmid constructions.

For expression in mammalian cells, the SIP1 (34) and δEF1 (5) cDNAs were subcloned into pCS3 (27). In this plasmid, the SIP1 and δEF1 open reading frames are fused to a $(Myc)_6$ tag at the N-terminus. SIP1 cDNA was also cloned into pCDNA3 (Invitrogen) as a N-terminal fusion with the FLAG tag. For the expression of $SIP1_{NZF}$ and $SIP1_{CZF}$, we subcloned into pCS3 the cDNA fragments encoding amino acids 1 to 389 and 977 to 1214, respectively. $SIP1_{CZF}$ (as amino acids 957 to 1156) and $SIP1_{NZF}$ (amino acids 90 to 383) were also produced in *E. coli* as a GST fusion protein (in pGEX-5X-1, Pharmacia) and purified using the GST purification module (Pharmacia). Identical mutations to those made in AREB6 (10) were also introduced in the SIP1 zinc fingers. Mutagenesis of zinc fingers NZF3, NZF4, CZF2 and CZF3 involved substitution of their third histidine to a serine. These mutations were introduced using a PCR based approach with the following primers: $SIP1_{NZF3Mut}$, 5'-CCACCTGAAAGAATCCCTGA GAATTCACAG; $SIP1_{NZF4Mut}$, 5'-GGGTCCTACAGTTCATCTATCAGCAGCAAG; $SIP1_{CZF2Mut}$, 5'-CACCACCTTATCGAGTCCTCGAGGCTGCAC; $SIP1_{CZF3Mut}$, 5'-TCCTACTCGCAGTCCATGAATCACAGGTAC. The respective mutated clusters were recloned in full size SIP1 in pCS3 in order to produce in mammalian cells the mutated SIP1 proteins named NZF3mut, NZF4mut, CZF2mut and CZF3mut, respectively. Furthermore, these mutated clusters were subcloned into pGEX5-X2 (Pharmacia), and produced in *E.coli* as a GST fusion protein (GST-NZF3mut, GST-NZF4mut, GST-CZF2mut and GST-CZF3mut). All constructs were confirmed by restriction mapping and sequencing.

Cell culture and DNA transfection.

COS1 cells were grown in DMEM supplemented with 10% fetal bovine serum. Cells were transfected using Fugene according to the manufacturer's protocol (Boehringer Mannheim), and collected 30-48 hrs after transfection.

Gel retardation assay.

The Xbra-WT oligonucleotide covers the region from –344 to –294 of the Xbra2 promoter (16). The region between –412 to –352 of the α4-integrin promoter is present within the α4I-WT oligonucleotide (26). The Ecad-WT probe contains the region between -86 to -17 of the human Ecad promoter (2). The sequences of the upper strand of the wild types and mutated double-stranded probes are listed in Table 1. Double-stranded oligonucleotides were labeled with [^{32}P]-γ-ATP and T4 polynucleotide kinase (New England Biolabs). Total cell extracts were prepared from COS1 cells (25) transfected with different pCS3 vectors allowing synthesis of full length SIP1, full length δEF1, and different mutant forms of SIP1 (25), or coproduction of equal amounts of Myc-tagged SIP1 and FLAG-tagged SIP1. GST-SIP1 fusion proteins were purified from *E.coli* extract using the GST purification module (Pharmacia), and tested in gel retardation. The DNA binding assay (20 µl) was performed at 25°C, with 1 µg of COS1 total cell protein, 1 µg of poly dI-dC, 10 pg of ^{32}P-labeled double-stranded oligonucleotide (approx. 10^4 Cerenkov counts) in the δEF1 binding buffer described previously (30). For supershift experiments, the extracts were incubated with anti-Myc (Santa Cruz) or anti-FLAG (Kodak) antibodies. For competition, an excess of unlabeled double-stranded oligonucleotides was added together with the labeled probe. The binding reaction was loaded onto a 4% polyacrylamide gel (acrylamide/bis-acrylamide, 19:1) prepared in 0.5XTBE buffer. Following electrophoresis, gels were dried and exposed to X-Ray film. All experiments were repeated at least three times.

Methylation interference assay.

The upper and the lower strand of the Xbra-WT probe were labeled separately and annealed with excess of complementary DNA strand. The probes were precipitated and treated with di-methyl-sulfate (8). The methylated probe (10^5 Cerenkov counts) was incubated in a 10 X gel retardation reaction (see above) (200 µl final volume) with 10 µg of total cell extract from COS1 cells expressing either $SIP1_{FS}$ or $SIP1_{CZF}$. After 20 min. of incubation at 25°C, the products were loaded onto a 4% polyacrylamide gel, and electrophoresis was performed as for the gel retardation assay. Subsequently, the gel was

blotted onto DEAE-cellulose membrane; the transfer was performed at 100 V for 30 min. in 0.5XTBE buffer. The membrane was then exposed for one hour, and the bands corresponding to the $SIP1_{FS}$ (or $SIP1_{CZF}$) and the free probe were eluted at 65°C, using high salt conditions (1M NaCl, 20 mM Tris, pH7.5, 1 mM EDTA). The eluted DNA was precipitated and treated with piperidine (18). After several cyles of solubilization in water and evaporation of the liquid under vacuum, the resulting DNA pellet was dissolved in 10 µl of sequencing buffer (97.5 % deionized formamide, 0.3 % each Bromophenol Blue and Xylene Cyanol, 10 mM EDTA) and denatured for 5 min. at 85 °C. The same amount of counts (1,500 Cerenkov counts) for the free probe and the bound probe was loaded onto a 20% polyacrylamide-8M urea sequencing gel. The gel was run in 0.5XTBE for one hour at 2,000 V. Thereafter, the gel was fixed in 50% methanol/10% acetic acid and dried. The gel was then exposed for autoradiography.

Western blot analysis.

Transfected cells were washed with PBS-O (137 mM NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4), collected in detachment buffer (10 mM Tris pH7.5, 1mM EDTA, 10% glycerol, with protease inhibitors (Protease inhibitor Cocktail tablets, Boehringer Mannheim)) and pelleted by low spin centrifugation. The cells were then solubilized in 10 mM Tris, pH 7.4, 125 mM NaCl, 1% Triton X-100. For direct electrophoretic analysis, gel sample buffer was added to the cell lysates and the samples were boiled. For other experiments, lysates were first subjected to immunoprecipitation with either anti-Myc or anti-FLAG antibodies. Antibodies were added to aliquots of the cell lysates, which were incubated overnight at 4°C. The antibodies and the bound protein(s) of the cell lysate were coupled as a complex to protein A-Sepharose for 2 hours at 4 °C. The immunoprecipitates were washed 4 times in NET buffer (50 mM Tris pH 8.0, 150 mM NaCl, 0.1% NP40, 1 mM EDTA, 0.25% gelatin), resolved by SDS-polyacrylamide (7.5%) gel electrophoresis, and electrophoretically transferred to nitrocellulose membranes. Membranes were blocked for 2 hours in TBST (10 mM Tris pH 7.5, 150 mM NaCl, 0.1 % Tween-20) containing 3% (w/v) non-fat milk, and incubated with primary antibody (1µg/ml) for 2 hours, followed by

secondary antibody (0.5 µg/ml) linked to horseradish peroxidase. Immunoreactive bands were detected with an enhanced chemiluminescence reagent (NEN).

Xenopus laevis transgenesis and whole-mount *in situ* hybridisation

Xenopus embryos transgenic for *Xbra*2-GFP were generated as described previously (Kroll and Amaya, 1996), with the following modifications. A Drummond Nanoinject was used for injecting a fixed volume of 5 nl of spermnuclei suspension per egg, at a theoretical concentration of 2 nuclei per 5 nl. *Not*I was used for plasmid linearisation and nicking of sperm nuclei. Approximately 800 eggs were injected per egg extract incubation. The procedure resulted in a successful cleavage of the embryo, which rates between 10% and 30%. Of these, 50 to 80 % completed gastrulation and 20 to 30% developed further into normal swimming tadpoles, if allowed. The transgenic frequency, as analysed by expression, varied between 50 to 90%. Embryos were staged according to Niewkoop and Faber (Niewkoop and Faber, 1967). A minimum of 30 expressing embryos were analysed per construct and shown stage. Whole-mount *in situ* hybridisation for the GFP reporter gene was as described previously (Latinkic *et al.*, 1997). After colour detection, embryos were dehydrated and cleared in a 2:1 mixture of benzyl alcohol/ benzyl benzoate.

Table 1.

Oligo	Sequence	Spacing
Xbra-WT	ATCCAGGC**CACCT**AAAATATAGAATGATAAAGTGACC**AGGTG**TCAGTTCT	24
Xbra-D	----------A----------	
Xbra-E	TAAAGTGACC**AGGTG**TCAGTTCT	
Xbra-F	ATCCAGGC**CACCT**AAAATATAGAATGA	
Rdm + Xbra-E	CAATTTAGAGTACTGTGTACTTGGGAGTAAAGTGACC**AGGTG**TCAGTTCT	
Xbra-F + AREB6	ATCCAGGC**CACCT**AAAATATAGAATGAGGCTCAGAC**AGGTG**TAGAATTCGGCG	23
Rdm + AREB6	CAATTTAGAGTACTGTGTACTTGGGAGGGCTCAGAC**AGGTG**TAGAATTCGGCG	
Xbra-WT	ATCCAGGC**CACCT**AAAATATAGAATGATAAAGTGACC**AGGTG**TCAGTTCT	24
Xbra-J	CGA----------	
Xbra-K	---ACT----------	
Xbra-L	------TAA----------	
Xbra-M	---------CAA----------	
Xbra-N	------------GCC----------	
Xbra-O	----------------CCG----------	
Xbra-P	------------------CGC----------	
Xbra-Q	----------------------TCC----------	
Xbra-R	------------------------GTC----------	
Xbra-S	----------T----------	
Xbra-Z	------------------------------------T----------	
Xbra-WT	ATCCAGGC**CACCT**AAAATATAGAATGATAAAGTGACC**AGGTG**TCAGTTCT	24
Xbra-B	ATCCAGGC**CACCTA**___TATAGAATGATAAAGTGACC**AGGTG**TCAGTTCT	21
Xbra-C	ATCCAGGC**CACCTAAAA**TATAGAATGAT___GTGACC**AGGTG**TCAGTTCT	21
Xbra-U	ATCCAGGC**CACCTAAAA**TATA_____GTGACC**AGGTG**TCAGTTCT	14
Xbra-EE	TAAAGTGACC**AGGTG**TCAGTTCTTAAAGTGACC**AGGTG**TCAGTTCT	18
Xbra-ErE	AGAACTGA**CACCT**GGTCACTTTATAAAGTGACC**AGGTG**TCAGTTCT	20
Xbra-FrF	ATCCAGGC**CACCT**AAAATATAGAATATTCTATATTTT**AGGTG**GCCTGGAT	24
Xbra-V	ATCCAGGC**AGGTG**TAAATATAGAATGATAAAGTGACC**CACCT**ACAGTTCT	24
Xbra-W	ATCCAGGC**AGGTG**TAAATATAGAATGATAAAGTGACC**AGGTG**TCAGTTCT	24
α4I-WT	GCAGGGCA**CACCT**GGATTGCATTAGAATGAGACTCACTACCCAGTTC**AGGTG**TGTTGCGT	34
α4I-A	----------A----------	
α4I-B	----------T----------	
Ecad-WT	TGGCCGGC**AGGTG**AACCCTCAGCCAATCAGCGGTACGGGGGGCGGTGCTCCGGGGCT**CACCT**GGCTGCAG	44
Ecad-A	----------T----------	
Ecad-B	----------A----------	

Table 1. **List of all the probes used in this study**. The CACCT sequences have been highlighted in bold. The spacing (right column) is the number of nucleotides present between the two CACCT sequences. Underlined gaps correspond to deletions of nucleotides from the wild type probes. For many probes, only the residues that have been changed compared with the wild type probes have been indicated in order to facilitate interpretation of the introduced mutations.

The invention is hereunder further explained by non-limiting examples without being restrictive in the scope of the current invention.

EXAMPLES

Example 1

SIP1 has a structure similar to δEF1

SIP1 was recently isolated as a Smad-binding protein and binds Smad1, Smad 5 and Smad2 in a ligand-dependent fashion (in BMP and activin pathways) (34). SIP1 is a new memeber of the family of two-handed zinc finger/homeodomain transcription factors, which also includes vetebrate δEF1 and *Drosophila* Zfh-1(4, 5). Like these, SIP1 contains two widely separated zinc finger clusters. One cluster of four zinc fingers (3 CCHH and 1 CCHC fingers) is located at the N-terminal region of the protein and another cluster of three CCHH zinc fingers is present at the C-terminal region (Fig. 1A). Between SIP1 and δEF1, a high degree of sequence identity is apparent within the N-terminal zinc finger cluster (87 %) and the C-terminal zinc finger cluster (97%)(see Fig.1B), whereas the two proteins are less conserved in the regions outside the zinc finger clusters (34). Therefore, it is assumed that SIP1 and δEF1 would bind to very similar sequences. In addition, the N-terminal and C-terminal zinc finger clusters of δEF1 bind to very similar sequences, which contain the core CACCT consensus sequence (10). Within the N-terminal cluster, both $\delta EF1_{NZF3}$ and $\delta EF1_{NZF4}$ are the main determinants for binding to the CACCT consensus sequence, and $\delta EF1_{CZF2}$ and $\delta EF1_{CZF3}$ are required for the binding of the C-terminal cluster (10). Moreover, the $\delta EF1_{NZF3+NZF4}$ domain shows high homology (67 %) with the $\delta EF1_{CZF2+CZF3}$ domain and this may explain why these two clusters bind to similar consensus target sites on DNA (Fig.1C). All the residues essential for binding, and which are conserved between $\delta EF1_{NZF3+NZF4}$ and $\delta EF1_{CZF2+CZF3}$, are also conserved between $SIP1_{NZF3+NZF4}$ and $SIP1_{CZF2+CZF3}$. Taken together, these comparisons suggested that the N- and C-terminal zinc finger clusters of SIP1 would also bind to very similar target sequences.

Example 2

Two CACCT sites are necessary for the binding of SIP1 to the Xbra2 promoter

CACCT sites are necessary for the binding of SIP1 to the Xbra2 promoter. SIP1 binds to the *Xenopus* Xbra2 promoter and represses expression of Xbra2 mRNA when overexpressed in the *Xenopus* embryo (34). The Xbra2 promoter contains several CACCT sequences, two of which are localized in a region (-381 to -231) necessary for the induction by activin (16). These two sites, an upstream CACCT and a downstream AGGTG (i.e. 5'-CACCT on the other DNA strand) respectively, are separated by 24 bp. To further elucidate the binding requirements of SIP1 to these sites, a corresponding 50 bp-long oligonucleotide (Xbra-WT; for a list of all probes see Table 1) was used as a probe in electrophoretic mobility shift assays (EMSAs). The Xbra-D probe, that contains a mutation of the downstream AGGTG site to AGATG, was included also. A similar mutation was shown previously to abolish the binding of δEF1 to the κE2 enhancer (30). In addition, we also tested the downstream site (probe Xbra-E) and the upstream site (probe Xbra-F) independently as shorter probes. These probes were incubated with total extracts of COS cells expressing the Myc-tagged C-terminal zinc finger cluster of SIP1 ($SIP1_{CZF}$), the Myc-tagged N-terminal zinc finger cluster of SIP1 ($SIP1_{NZF}$), or Myc-tagged full size SIP1 ($SIP1_{FS}$).

When mock-transfected COS cells are used as control with the A probe, two weak complexes and one strong complex are visualized (Fig.2, lane 9). Using competitor oligonucleotides, the two weak complexes turned out to be non-specific, whereas the strong, fast migrating complex shows specificity for binding to the Xbra probe. The latter observation suggests that COS cells contain an endogenous protein that can bind to the Xbra-WT probe. When $SIP1_{CZF}$ is present in the extract, we observed a strong and slow migrating complex (lane 1), in addition to the endogenous binding activity from the COS extract (compare lane with 9). This complex could be supershifted with an anti-Myc antibody, which confirms that it results from binding of $SIP1_{CZF}$ to the Xbra-WT probe. Mutation of the downstream site (Xbra-D probe) strongly affected the formation of this $SIP1_{CZF}$ complex (lane2). Moreover, $SIP1_{CZF}$ binds to the Xbra-E probe (lane 3), but not to

the Xbra-F probe (lane 4) indicating that the downstream site is essential for binding of $SIP1_{CZF}$, and $SIP1_{CZF}$ may exclusively bind to this site. The strong complex visualized with the Xbra-F probe was also present in $SIP1_{FS}$ extracts (lane 8) and in mock extract, and originates from hitherto uncharacterized endogenous COS cells protein binding to the Xbra-F probe. In addition, COS cell extracts containing $SIP1_{NZF}$ displayed similar binding patterns in EMSAs as obtained with $SIP1_{CZF}$. It is apparent that, like in δEF1 (10), both zinc finger clusters of SIP1 have similar DNA binding features.

A strong complex, corresponding to $SIP1_{FS}$, is also generated with the Xbra-WT probe (lane 5). It is important to mention that the $SIP1_{CZF}$ production level in COS cells is approximately 50-fold higher than the $SIP1_{FS}$ level . For each EMSA reaction, we always used the same amount of crude COS cell proteins. The binding of $SIP1_{FS}$ to Xbra-WT probe is as strong as the binding of $SIP1_{CZF}$. Interestingly, this indicates that the affinity of $SIP1_{FS}$ for Xbra-WT is at least 50 times higher than this of $SIP1_{CZF}$.

The $SIP1_{FS}$ complex, similar to $SIP1_{CZF}$ and $SIP1_{NZF}$, is absent when using the mutated Xbra-D probe (lane 6). Thus, an intact downstream site is again required for the binding of $SIP1_{FS}$. In contrast to $SIP1_{CZF}$ and $SIP1_{NZF}$, which bind with similar affinities to the Xbra-WT and Xbra-E probes, $SIP1_{FS}$ does not bind to the Xbra-E probe (lane 7, compare with lane 3). Like $SIP1_{CZF}$ and $SIP1_{NZF}$, $SIP1_{FS}$ does not bind to the Xbra-F probe. We conclude that the downstream site (AGGTG) is necessary for $SIP1_{FS}$ to bind to the Xbra2 promoter. However, this site is not sufficient because additional sequences upstream of the Xbra-E probe are necessary for the binding of $SIP1_{FS}$. One of the reasons for which $SIP1_{FS}$ was unable to bind to the Xbra-E probe may simply be the length of the Xbra-E probe, because it is shorter than the Xbra-WT probe. To test this, we prepared a probe containing a random sequence (Rdm) upstream of the Xbra-E probe (Table 1) in order to extend it to the same length as Xbra-WT. In contrast to $SIP1_{CZF}$, which bound efficiently to Rdm+Xbra-E probe (see Fig.3A, lane 6), $SIP1_{FS}$ was unable to bind (lane 3). This result demonstrates that length of the Xbra-E probe per se is not the cause of the failure of $SIP1_{FS}$ to bind to this probe.

To substantiate that the Xbra-F oligonucleotide also contains sequences necessary for the

binding of $SIP1_{FS}$, we fused this oligonucleotide as well as a random sequence upstream of another CACCT site known to be bound strongly by AREB6 protein (10) (probes Xbra-F + AREB6 and Rdm + AREB6, respectively). As shown in Fig.3A, $SIP1_{CZF}$ bound, with equal affinity, both the Xbra-F + AREB6 and Rdm + AREB6 probes (lanes 4 and 5), indicating that the AREB6 sequence is also recognized by $SIP1_{CZF}$. However, $SIP1_{FS}$ only binds to the Xbra-F + AREB6 probe (lane 1) but not to Rdm + AREB6 (lane 2). This confirms that the Xbra-F oligonucleotide contains sequences necessary for the binding of $SIP1_{FS}$. In addition, the only common feature between the Xbra-E and the AREB6 probe is the CAGGTGT sequence, suggesting that no other sequences than this CAGGTGT in the Xbra-E probe are necessary for the binding of $SIP1_{FS}$. One of the reasons for which $SIP1_{FS}$ is unable to bind to the Xbra-E probe might be because the length of the Xbra-E probe is shorter than the length of the Xbra-WT probe. To test this hypothesis, we prepared a probe containing a random sequence upstream of the Xbra-E probe to obtain the same length as the Xbra-WT probe. In contrast to $SIP1_{CZF}$ that binds efficiently to this probe (Fig.2, lane 6), $SIP1_{FS}$ was unable to bind (lane3). This result clearly indicates that the length of the Xbra-E probe was not the reason for which $SIP1_{FS}$ does not bind to this probe. To substantiate that the Xbra-F oligonucleotide also contains sequences necessary for the binding of $SIP1_{FS}$, we fused that oligonucleotide as well as a random sequence upstream of another CACCT site known to bind strongly AREB6 protein (Xbra-F + AREB6 and Rdm + AREB6, respectively). In lanes 4 and 5, we observed that SIP_{CZF} binds with equal affinities both the Xbra-F + AREB6 and Rdm + AREB6 probes, indicating that the AREB6 sequence is also recognized by $SIP1_{CZF}$. However, $SIP1_{FS}$ only binds to the Xbra-F + AREB6 probe (lane 1) and not to the Rdm + AREB6 probe. This confirms that the Xbra-F oligonucleotide contains sequences necessary for the binding of $SIP1_{FS}$. In addition, the only common denominator between the Xbra-E and the AREB6 probe is the AGGTG sequence, suggesting that no other sequences than this AGGTG in the Xbra-E probe is necessary for the binding of $SIP1_{FS}$.

To map the sequences within Xbra-F that, in conjunction with the Xbra-E sequence, are required for the binding of $SIP1_{FS}$, we prepared a series of probes, identical in length to Xbra-WT, containing adjacent triple mutations within the Xbra-F part (see Table 1). Only

three of these mutated probes (i.e. Xbra-L, Xbra-M and Xbra-N) affected the binding of $SIP1_{FS}$ (Fig.3B). Indeed, the upstream CACCT sequence, which is intact in the Xbra-F probe, was modified in the L, M and N probes. We also showed that $SIP1_{FS}$ does not bind to the Xbra-S probe, which contains a point mutation, changing the upstream CACCT into CATCT. This mutation is similar to the downstream AGATG mutation made within the Xbra-D probe.

The results described above are indicative for $SIP1_{FS}$ contacting both CACCT sequences in the Xbra promoter. To further investigate the importance of these sites, a DNA methylation interference assay was carried out (Fig. 3B). The methylation of three Gs of the downstream AGGTG (SIP_{DO}) and of the two Gs of the upstream CACCT (SIP_{UP}) was significantly lower in the $SIP1_{FS}$ bound versus unbound probe, suggesting that the methylation of these Gs interfered with the binding of $SIP1_{FS}$. This strongly supports that these residues are essential for $SIP1_{FS}$ binding. It has also been observed that the methylation of one of the 2 Gs localized very close to the SIP_{DO} also interfered with the binding of $SIP1_{FS}$ (middle lane, right panel). Consequently it has thus been shown that for $SIP1_{FS}$ two CACCT sequences and their integrity are required for DNA binding.

Example 3

SIP1 and δEF1 require 2 CACCT sequences for binding to different potential candidate sites.

SIP1 and δEF1 have a very similar structure with two very highly conserved zinc finger clusters and it is likely that these two proteins bind DNA in a similar way. We set out whether also δEF1 binds to the Xbra2 promoter by contacting both CACCT sequences, which has previously not been reported. Myc-tagged δEF1 was expressed in COS cells and the corresponding nuclear extracts were tested in EMSA with WT and a panel of mutated Xbra probes (Fig.4, panel A). δEF1 binds strongly to the Xbra-WT probe (lane 1) that contains both CACCT sites. However, like $SIP1_{FS}$, δEF1 binds neither the Xbra-E probe comprising only the downstream CACCT site (lane 4) nor the Xbra-F probe containing only

the upstream CACCT site (lane 5). In addition, the point mutation of either the upstream CACCT (Xbra-S) or the downstream CACCT site (Xbra-D) also abolished the binding of δEF1. Therefore, like $SIP1_{FS}$, full length δEF1 requires also the integrity of both CACCT sequences for binding to the Xbra2 promoter. The fact that two CACCT sites are required for the binding of $SIP1_{FS}$ as well as δEF1 may be unique for the Xbra2 promoter. Therefore, the next question was to analyse whether two CACCT sequences are also necessary for SIP1/δEF1 for binding to other target sites. Putative δEF1 and SIP1 binding elements are present in several promoters. One putative δEF1 binding element, indeed containing two intact and spaced CACCT sites, was found within the promoter of the human α4-integrin gene (23). Interestingly, both sites are cointained wihtin of E2 boxes. Mutation of these two CACCT sites led to the derepression of the α4-integrin gene expression in myoblasts, suggesting that δEF1 is a repressor of α4-integrin gene transcription (23). Since these two CACCT sites are closely positioned in the promoter (spacing is 34 bp), we investigated whether both CACCT sequences are required for the binding of δEF1. For this purpose, a 60 bp-long probe overlapping both CACCT sites of the α4-integrin promoter was synthesized (α4I-WT) as well as two mutated versions, i.e. having a point mutation in either the upstream (α4I-B) or the downstream CACCT site (α4I-A), respectively (see Table 1). These probes were tested for binding in EMSAs with COS cell extracts of either δEF1 or $SIP1_{FS}$ transfected cells (Fig.4, panel B). Both δEF1 (lane 4) as well as $SIP1_{FS}$ (lane 1) form strong complexes with the α4I-WT probe. The δEF1 complex was entirely supershifted with an anti-Myc antibody (lane 7), demonstrating its specificity. Both the binding of SIP1 and of δEF1 is abolished or strongly affected by a mutation of either the upstream or the downstream CACCT site (lanes 2-3 and 5-6). Moreover, competition experiments (Fig.4, panel C) revealed that 50 ng of unlabeled α4I-WT probe was sufficient to abolish the binding of SIP1 or δEF1 to the α4I-WT probe, whereas 50 ng of either unlabeled α4I-A or α4I-B probes were not. We conclude that $SIP1_{FS}$ as well as δEF1 require the integrity of two CACCT sites for binding to the promoter of the α4-integrin gene.

We also found two closely positioned CACCT sites within the promoter of the human E-

cadherin gene. An oligonucleotide comprising both CACCT sites of this E-cadherin promoter was used as a probe (Ecad-WT) together with $SIP1_{FS}$ or δEF1 extracts in EMSAs (Fig.4, panel D). Both $SIP1_{FS}$ as well as δEF1 form a complex with this probe. However, when either the upstream (Ecad-A probe) or the downstream (Ecad-B probe) CACCT site was mutated (see Table 1, lower part), the binding of $SIP1_{FS}$ and δEF1 was abolished. This also suggests that the two CACCT sites in this promoter represent a high affinity site for the binding of two-handed zinc finger/homeodomain transcription factors.

From the alignment of the Xbra-WT, α4I-WTand Ecad-WT probes (see Table 1) we observed no obvious homology, except for one CACCTG site and a second CACCT site. Our results described above and this alignment, indicates that only those sequences participate in the binding of either $SIP1_{FS}$ or δEF1. We therefore conclude that for binding to target promoters, $SIP1_{FS}$ or δEF1 require at least one CACCT site and one CACCTG site.

Example 4

Spacing variations and orientation of the CACCT sites

Within the Xbra-WT, α4I-WT and Ecad-WT probes (Table 1), the spacing between the two CACCT sequences was 24 bp, 34 bp and 44 bp, respectively. Since $SIP1_{FS}$ and δEF1 bind efficiently to these probes, this shows that these proteins can accommodate spacing between the two CACCT sites ranging from 24 bp to at least 44 bp. To further investigate whether the spacing between the two CACCT sites is an important parameter for binding, we generated different Xbra probes with deletions between these sites. Two mutant probes (Xbra-B and Xbra-C) have a deletion of 3 adenines whereas probe Xbra-U has a deletion of 10 nucleotides (Table 1). These probes were tested in EMSA with cell extracts from COS cells expressing either $SIP1_{FS}$ or δEF1 (Fig. 5). Both $SIP1_{FS}$ and δEF1 bind with equal affinity to the Xbra-WT, Xbra-B, Xbra-C and Xbra-U probes (lanes 1 to 4). As already suggested by the results shown for different promoters in Fig. 4, this indicates that also within the same promoter element, the spacing between the two CACCT sites is not a critical parameter for the binding of these two transcription factors.

By extensive comparison of the Xbra-WT, α4I-WT and Ecad-WT probes, we observed that

in the case of the Xbra-WT and α4I-WT probes, the orientation of the two CACCT sites is CACCT-N-AGGTG, whereas in Ecad-WT the orientation is AGGTG-N-CACCT. Because of the non-palindromic feature of the CACCT site, these two sites could be assumed substantially different. However, $SIP1_{FS}$ and δEF1 bind to these differentially orientated sites with comparable affinities (see above). This suggests that $SIP1_{FS}$ and δEF1 can bind irrespective of the orientation of the two CACCT sites.

To further investigate the orientation of the two CACCT sites with respect to the DNA binding capacity of $SIP1_{FS}$ and δEF1, additional probes were designed. Probe Xbra-EE contains a tandem repeat of the Xbra-E probe, whereas probe Xbra-ErE contains an inverted repeat of the same Xbra-E sequence. In addition, we synthesized Xbra-V, in which the upstream CACCT site (plus one extra base pair on each side) was replaced by the downstream AGGTG sequence and vice versa. Finally, in the Xbra-W probe, only the downstream site was replaced by the upstream CACCT sequence. All these probes were again tested in EMSAs with extracts prepared from COS cells expressing either $SIP1_{FS}$ or δEF1 (Fig. 5). We observed the strongest binding of $SIP1_{FS}$ or δEF1 to the Xbra-EE probe (lane 5). Therefore, $SIP1_{FS}$ and δEF1 cannot bind to Xbra-E, containing a single CACCT site, but bind strongly when this sequence is duplicated, again indicating the requirement for 2 CACCT sites. In addition, it is evident that the two CACCT sites have to be present on the same DNA fragment and not on two separated strands (see below and lane 10). SIP1 and δEF1 bind to Xbra-ErE, also suggesting that the respective orientation of the two CACCT sites is not critical for binding. Furthermore, switching both the upstream and the downstream sites (probe Xbra-V) or replacing only the upstream site by a second copy of the downstream site (probe Xbra-W) did not have an effect on $SIP1_{FS}$ and δEF1 binding. From these experiments, we conclude that neither the spacing between the two CACCT sites nor the respective orientation of these two sites is critical for the binding of two-handed zinc finger/homeodomain transcription factors *in vitro*.

Surprisingly, not all CACCT duplicated sites can bind these factors. In fact, duplication of the Xbra-F sequence, which in combination with the Xbra-E sequence was shown to be necessary for the binding of $SIP1_{FS}$ and δEF1, is refractory to binding of $SIP1_{FS}$ and δEF1

(Fig.5, lane 9 for the inverted repeat (Xbra-FrF). This suggests that the CACCT site within the Xbra-F context is a low affinity site and that sequences adjacent to this CACCT site may optimize the affinity. In addition, the fact that neither the C-terminal cluster (Fig.2) nor the N-terminal cluster can bind independently to the Xbra-F probe confirms the assumption that this site displays low affinity. In contrast, the CACCTG site present in the Xbra-E probe can bind $SIP1_{CZF}$ and $SIP1_{NZF}$, and a duplication of this element creates a high affinity-binding site for both $SIP1_{FS}$ and full length δEF1 (lanes 5). This suggests that the terminal G base in the downstream site may also allow to discriminate between a high and low affinity-binding site. However, the CACCT site in Xbra-F may only bind one of the zinc finger clusters of $SIP1_{FS}$ once the other cluster has occupied the neighboring high affinity CACCTG site (in Xbra-E). To confirm the importance of this terminal G base residue for the binding of $SIP1_{FS}$ and δEF1, we mutagenized the downstream CACCTG site to CACCTA (probe Xbra-Z). The binding of $SIP1_{FS}$ or δEF1 to the Xbra-Z probe was strongly decreased (compared with the Xbra-WT probe) suggesting that this G-base residue is important for the generation of a high affinity binding site for both $SIP1_{FS}$ and δEF1.

Finally, when Xbra-E and Xbra-F probes are mixed prior to addition of $SIP1_{FS}$ or δEF1, we do not observe any binding, again indicating that both CACCT sites have to be in the *cis* configuration, i.e. on the same DNA (Fig.5, lane 10).

Example 5

The two zinc finger clusters of SIP1 are required and must be intact for binding to DNA

SIP1 and δEF1 bind to DNA elements containing two CACCT sites and both of these proteins contain two clusters of zinc fingers capable of binding independently to CACCT sites. In subsequent work, we wanted to evaluate the importance of each zinc finger cluster for the binding of $SIP1_{FS}$ to DNA. Mutations destroying either the third or the fourth zinc finger of the N-terminal cluster of $\delta EF1_{NZF}$ were shown to abolish the binding of this cluster to the DNA. Similarly, mutagenesis of the second or the third zinc finger in the C-terminal cluster also abolished the binding of $\delta EF1_{CZF}$ to CACCT (10). Therefore, we introduced in

the $SIP1_{NZF}$ and $SIP1_{CZF}$ clusters mutations similar to those in δEF1. These mutated and wild type clusters were fused to GST and the fusions proteins were purified from bacteria. Figure 6 (panel A) shows that both wild type $SIP1_{NZF}$ (lane 1) and $SIP1_{CZF}$ (lane 4) strongly bind to the Xbra-E probe. However, with the same amount of purified mutant cluster/GST fusion proteins (GST-NZF3, GST-NZF4, GST-CZF2 and GST-CZF3), no binding to the Xbra-E probe could be detected with any of these fusion proteins (lanes 2, 3, 5 and 6). Indeed, these mutations also abolish the capacity of each cluster ($SIP1_{NZF}$ and $SIP1_{CZF}$) to bind independently to a CACCT site.

Then, we introduced similar mutations in full size SIP1 (NZF3-Mut, NZF4-Mut, CZF2-Mut and CZF3-Mut), and overexpressed these SIP1 mutants in COS cell as Myc-tagged proteins. The expression of the different mutants was established and normalized by Western blot analysis using anti-Myc antibody (Fig.6, panel D). By means of EMSAs (Fig. 6, panel B), we observed that WT SIP1 binds strongly to the Xbra-WT probe (lane 1), and that the SIP1-complex is supershifted upon incubation with an anti-Myc antibody (lane 6). In contrast, none of the mutant forms of full size SIP1 was able to form a SIP1-like complex (lanes 2 to 5) or a SIP1 supershifted complex (lanes 7 and 8). The same observations were made when the αI4-WT probe was used as a probe (Figure 6, panel C). In conclusion, full size SIP1 requires the binding capacities of both intact zinc fingers clusters to bind to its target, which necessarily contains 2 CACCT sites. The effect of these mutations on the repressor activity of SIP1 was tested in a transfection assay together using p3TP-Lux reporter plasmid. This plasmid contains three copies, each of which has one CACCT, of a sequence covering the –73 to –42 region of human collagenase promoter (de Groot and Kruijer, 1990). SIP1 bound to a fragment containing this mutimerized element (Fig.9A), but neither NZF3-Mut nor CZF3-Mut was able to bind. Overexpression of SIP1 in CHO cells leads to a strong repression of the p3TP-Lux basal transcriptional activity. However, the repression was 6 to 7-fold lower upon overexpression of SIP1 mutants defective in DNA binding (NZF3-Mut or CZF3-Mut) (Fig.9B). Therefore the integrity of both zinc finger clusters is necessary for both the DNA-binding and optimal, i.e. wild-type repressor activity of SIP1.

Example 6

SIP1 binds to DNA as a monomer

The observation that the integrity of both SIP1 zinc fingers clusters is required for its binding to two CACCT sequences, prompted us to test whether SIP1 binds as a monomer, in which each zinc finger cluster contacts one CACCT site. However, it can be hypothesized also that SIP1 binds to its target sites as a dimer. This may imply that one of the SIP1 proteins of the dimer would bind one CACCT site via its N-terminal zinc finger cluster, while the second SIP1 molecule would contact the DNA via its C-terminal zinc finger cluster. Consequently, certain combinations of NZF and CZF mutants in a full size SIP1 context (see above) should generate a dimeric configuration that binds DNA. As shown already in Figure 6B, in none of the combinations of NZF with CZF mutations tested, binding to the Xbra-WT probe could be detected. Although we cannot rule out that these mutations also would affect dimer formation, it is highly unlikely that the same mutation affects both the DNA binding capacity as well as the monomer-monomer interaction. Moreover, it is highly unlikely that two different mutants, i.e. different mutations within a cluster, would behave identical. Therefore, we considered that SIP1 does not bind to DNA as a dimer.The observation that the integrity of both zinc fingers clusters is required for SIP1 binding to two CACCT sequences, suggests that SIP1 binds as a monomer, in which each zinc finger cluster contacts one CACCT site. However, it can be hypothesized that SIP1 binds its target sites as a dimer. This would imply that one of the SIP1 molecules of the dimer would bind one CACCT site via its N-terminal zinc finger cluster, while the second SIP1 molecule would contact the DNA via its C-terminal zinc finger cluster. Since both zinc finger clusters are necessary for binding, the zinc finger cluster not interacting with the DNA would then be involved in dimerization. Consequently, some combinations of NZF and CZF mutants (see above) should generate a dimer configuration that binds DNA. As shown in Figure 5A, in none of the combinations of NZF and CZF mutations binding to the Xbra-WT probe could be detected. Although we cannot rule out that these mutations also affect potential dimer formation, it is highly unlikely that the same mutation affects both the DNA-binding capacity as well as the protein-protein interaction. Moreover, it is highly unlikely that

two different mutants, ie have different mutations within a cluster, would behave the same. These observations indicate that SIP1 does not bind DNA as a dimer.

To address this experimentaly, we used a combination of differently tagged SIP1 with supershift experiments in EMSAs. First, we produced Myc-tagged and/or FLAG-tagged $SIP1_{FS}$ separately at comparable levels in COS cells, and confirmed that both proteins bind to DNA with similar affinities. The SIP1 complex generated with Myc-tagged SIP1 has a slightly slower migration than the FLAG-tagged complex (the Myc-tag is longer than the FLAG-tag). Extracts prepared from COS cells expressing similar amounts of both Myc-tagged and FLAG-tagged SIP1 were incubated with the Xbra-WT probe and used in EMSAs. In figure 7, lane 1, we observed the formation of a broad SIP1 complex which is a combination of both the fast migrating FLAG-tagged SIP1 complex with the slow migrating Myc-tagged SIP1 complex. Using an anti-FLAG antibody, only the lower part of the complex corresponding to FLAG-tagged SIP1 is supershifted, whereas about 50 % of the radioactivity remains within the Myc-tagged SIP1complex. This indicates that the latter SIP1 complex is not supershifted with the anti-FLAG antibody. Conversely, incubating the extract with an anti-Myc antibody supershifted only the lower part of the complex corresponding to Myc-tagged SIP1 whereas 50% of the radioactivity is retained within the FLAG-tagged SIP1 complex. Again, this indicates that no FLAG-tagged SIP1 is supershifted with an anti-Myc antibody. Using both antibodies, we observed the same two supershifted bands, which correspond to the Myc-tagged and the FLAG-tagged supershifted complex, in the upper part of the gel. If SIP1 dimers would be formed, then at least some heterodimers would be assembled from Myc-tagged SIP1 and FLAG-tagged SIP1. However, no other supershifted band that would correspond to a potential double supershift, viz. supershifted with both anti-Myc- and anti-FLAG-antibodies, is detectable. Hence, this experiment gave no detectable dimer formation between FLAG-tagged SIP1 and Myc-tagged SIP1.

Finally, FLAG-tagged SIP1 in a COS cell extract was immunoprecipitated in the presence of a large excess of DNA binding sites. However, co-immunoprecipitation of Myc-tagged SIP1 was not feasible. The reciprocal experiment, i.e. immunoprecipitating with an anti-Myc antibody and detection with an anti-FLAG antibody, did not show any SIP1 dimer either.

Taken together, these observations let us to conclude that SIP1 binds as a monomer to the Xbra-WT probe.

Example 7

Mutations in either the upstream or downstream CACCT lead to ectopic activity of the Xbra2 promoter in transgenic frog embryos

SIP1 binds to the *Xbra2* promoter and represses expression of endogenous Xbra2 mRNA when overexpressed in *Xenopus* embryos (Verschueren *et al.*, 1999). To analyse the importance of CACCT sequences in the regulation of the *Xbra2* promoter *in vivo*, we tested whether mutations of these would affect *Xbra2* promoter activity in transgenic embryos. *Xbra2* promoter sequences were fused upstream of the Green Fluorescent Protein (GFP) gene and this reporter cassette was used for transgenesis. A 2.1 kb-long *Xbra2* promoter fragment was shown sufficient to yield the reporter protein synthesis in the same domain of the embryo (85% of the embryos, stage 11, n=57) as compared with endogenous Xbra mRNA (which is in the marginal zone) except in the organizer region, for which a regulatory element may be lacking in the reporter cassette tested here (a more detailed spatial and temporal analysis of other putative regulatory elements and the SIP1/δEF1 binding site of the *Xbra2* promoter *in vivo* will be submitted elsewhere, Lerchner *et al.*, in preparation).

A single point mutation within the downstream CACCT site in the promoter, which disrupted SIP1 binding (Xbra2-Mut1; Fig.10A, lane 2) and is identical to XbraD, had a severe effect on spatial production of the reporter protein. All embryos (n>30) showed ectopic expression in the inner ectoderm layer (Fig.10B). Mutations within the upstream CACCT sequence (Xbra2-Mut4) also affected the SIP1 binding (Fig.10A, lane 3): we observed in all transgenic embryos (n>30) the same ectopic expression as for the Xbra2-Mut1 mutation (Fig.10B). Mutation of the downstream CACCTG to CACCTA (Xbra2-Mut2) also affects SIP1 binding to such probe (Fig. 10A, lane 4). This mutation when introduced into the *Xbra2* 2.1kb promoter also led to ectopic expression of GFP mRNA in all transgenic embryos tested (n>30; Fig.10B). We also tested a mutation (Xbra2-Mut3) that decreased by 3 bp the original 24 bp-spacing between the two CACCT sequences. This mutation

weakened the interaction of such probe with SIP1 (Fig.10A, lane 5). This was also reflected in the corresponding transgene embryos (n=37): while 35% of the embryos showed the same expression pattern as the wild type *Xbra2* 2.1kb promoter fragment, 65% had either patches or weak continuous expression in the inner ectoderm layer (Fig.10B).

A nice correlation between the effect of these mutations on SIP1 binding affinity in EMSA and the phenotype (ectopic expression of the reporter gene) and its penetrance *in vivo* was thus obtained, indicating the importance of the SIP1 target sites in the normal regulation of *Xbra2* expression in *Xenopus* development (stage 11). It also suggests that an hitherto unknown *Xenopus* SIP1-like repressor regulates *Xbra2* gene expression *in vivo*. In addition, it confirms that SIP1-like factors require two intact CACCT sites for regulating target promoters like *Xbra2*.

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Brief description of the figures

Figure 1. **Schematic representation of Zfh-1, SIP1 and δEF1, and alignment of the SIP1 and δEF1 zinc fingers.** (A) Schematic representation of mouse δEF1 (1117 amino acids) and SIP1 (1214 amino acids). The filled boxes represent CCHH zinc fingers, the open boxes are CCHC zinc fingers. The homeodomain-like domain (HD) is depicted as an oval. The percentage represents the homology between different domains. SIP1 polypeptides used in this study are depicted with their coordinates. SBD: Smad-binding domain (Verschueren *et al.*, 1999). (B) Alignments of the amino acid sequences from zinc fingers of SIP1 and δEF1. Vertical bars indicate sequence identity. The conserved cysteine and histidine residues forming the zinc fingers are printed in bold, and indicated by an asterisk. The residues in zinc fingers that can contact DNA are indicated with an arrow. (C) Alignment of the protein sequence of $SIP1_{NZF3+NZF4}$ and $SIP1_{CZF2+CZF3}$, and of $\delta EF1_{NZF3+NZF4}$ and $\delta EF1_{CZF2+CZF3}$, respectively, demonstrating intramolecular conservation of zinc fingers.

Figure 2. **Gel retardation assay with different probes from the Xbra2 promoter.** The different Xbra ^{32}P labeled probes (10 pg) were incubated with 1 µg of total protein extract from COS1 cells transfected with pCS3-$SIP1_{CZF}$ (lanes 1 to 4), with pCS3-$SIP1_{FS}$ (lanes 5 to 8) or from mock-transfected cells (lane 9). The $SIP1_{CZF}$ specific complexes are indicated with grey arrows and the $SIP1_{FS}$ specific complex is indicated with a black arrow (lane 5). All other complexes are generated from DNA-binding activities present in mock-transfected COS1 cells.

Figure 3. **Two CACCT sites are contacted upon binding of $SIP1_{FS}$ to the Xbra2 promoter.**

(A) Only mutations within the upstream CACCT sequence (as revealed by scanning mutagenesis, see Table I) or the downstream CACCT sequence (see elsewhere in Table I) of XbraWT abolish $SIP1_{FS}$ binding. (B) Methylation interference assay indicates that $SIP1_{FS}$ contacts both CACCT sequences. XbraWT either labeled in the upper (left panel) or the

lower (right panel) strand were methylated and incubated with total extract from COS1 cells transfected either with pCS3-SIP1$_{FS}$ or pCS3-SIP1$_{CZF}$. The DNA retarded in the shifted complex or the unbound DNA (FREE) were purified, cleaved with piperidine and run onto a sequencing gel. The arrows indicate the guanine residues that are methylated in the free probe. SIP$_{UP}$ and SIP$_{DO}$ indicate the upstream and the downstream CACCT from the *Xbra2* promoter, respectively.

Figure 4. **Two CACCT sequences are necessary for the binding of SIP1$_{FS}$ and δEF1 to the Xbra2, the α4-integrin and the E-cadherin promoters.** (**A**) δEF1 binding to the Xbra2 promoter. (**B**) SIP1 and δEF1 binding to the α4-integrin promoter. (**C**) Binding of SIP1 and δEF1 to the α4-integrin promoter, including competition with excess of non-labeled wild type and mutated binding sites. (**D**) Binding of SIP1 and δEF1 to the E-cadherin promoter. In each binding reaction, 10 pg of labeled probes were incubated with 1 µg of a total cell protein extract prepared from COS1 cells transfected with either pCS3-SIP1$_{FS}$ or pCS3-δEF1. In the competition experiments, 5 ng and 50 ng of unlabeled DNA was added at the same time as the labeled probe. In lane 7, panel B we added Myc-tag directed antibody to the binding reaction and the supershifted complex is indicated by an asterisk (*). The black (▲) arrows and squares (◆) indicate the δEF1 and the SIP1 retarded complex, respectively. For the sequences of all probes, see Table1).

Figure 5. **The spacing and the relative orientation of the CACCT sequences are not critical for the binding of SIP1$_{FS}$ and δEF1 to the Xbra2 promoter.** Ten pg of labeled probes were incubated with 1 µg of a total cell protein extract prepared from COS1 cells transfected with either pCS3-SIP1$_{FS}$ or pCS3-δEF1. In lane 10 we used 10 pg of the Xbra-E probe and 10 pg of the Xbra-F probe in the same binding reaction. For reasons of clear and comparative presentation, we omitted the free probe from the SIP1 binding reactions.

Figure 6. **The integrity of both SIP1 zinc finger clusters is necessary for the binding of SIP1$_{FS}$ to DNA.** (**A**) Mutations within NZF3, NZF4, CZF2, CZF3 abolish the DNA-binding

activity of either the $SIP1_{NZF}$ or $SIP1_{CZF}$ zinc finger clusters. The wild type and mutated zinc finger clusters were fused to GST and the fusion proteins were produced in *E.coli*. After purification, an equal amount of each fusion proteins (0.1 ng) was incubated with 10pg of labeled Xbra-E probe. (**B**) Mutations within NZF3, NZF4, CZF2 or CZF3 affect the binding of $SIP1_{FS}$ to the Xbra-WT probe. Ten pg of labeled Xbra-WT probe were incubated with 1 µg of a total cell protein extract prepared from COS1 cells transfected with either pCS3-$SIP1_{FS}$ (lanes 1 and 6), pCS3-$SIP1_{NZF3mut}$ (lanes 2 and 7), pCS3-$SIP1_{NZF4mut}$ (lane 3), pCS3-$SIP1_{CZF2mut}$ (lane 4), pCS3-$SIP1_{CZF3mut}$ (lanes 5 and 8). In lanes 9 to 14, all possible combinations of 2 COS cell extracts (1 µg of each) expressing different of SIP1 mutants were tested. In lanes 6 to 8, we added Myc-tag directed antibody to the binding reaction and the supershifted complex is indicated with by an asterisk (*). The arrow indicates the $SIP1_{FS}$ retarded complex. (**C**) Mutations within NZF3, NZF4, CZF2 or CZF3 abolish the binding of $SIP1_{FS}$ to the α4-integrin promoter. Ten pg of labeled α4I -WT probe were incubated with 1 µg of a total cell protein extract prepared from COS1 cells transfected with either pCS3-$SIP1_{FS}$ (lanes 1 and 6), pCS3-$SIP1_{NZF3mut}$ (lanes 2 and 7), pCS3-$SIP1_{NZF4mut}$ (lane 3), pCS3-$SIP1_{CZF2mut}$ (lane 4), pCS3-$SIP1_{CZF3mut}$ (lanes 5 and 8). In lanes 6 to 8, we added Myc-tag directed antibody to the binding reaction and the supershifted complex is indicated with an asterisk (*). The arrow indicates the $SIP1_{FS}$ retarded complex. (**D**) SIP1 mutants are produced in comparable amounts in COS cells. Ten µg of the COS cell total extract were analyzed by Western blotting using the anti-Myc antibody. SIP1 mutant expression levels are in fact slightly higher that SIP1-WT expression level.

Figure 7. **$SIP1_{FS}$ binds as a monomer to the Xbra-WT probe.** In lanes 1 to 4, 10 pg of labeled Xbra-WT probe were incubated with 1 µg of total cell protein prepared from COS1 cells transfected with an equal amount of pCS3-$SIP1_{FS}$ (Myc-tagged) and of pCDNA3-SIP1 (Flag-tagged). In lanes 2 and 3 we added anti-Flag and anti-Myc antibodies, respectively. Both anti-Flag and anti-Myc antibodies were added to the binding assay in lane 4. The Flag- and the Myc-supershifted complexes are indicated with an asterisk and a bullet, respectively.

Figure 8. **Possible DNA-binding mechanisms for SIP1.** Model 1 : SIP1 binds DNA as a monomer. Model 2 : SIP1 binds DNA as a dimer.

Figure 9. **The integrity of CZF or NZF is necessary for SIP1 repressor activity**. (A) $SIP1_{FS}$ binding to a gel-purified fragment derived from the multiple CACCT-containing artificial promoter from reporter plasmid p3TP-Lux. In lane 2, we added anti-Myc tag antibody; the supershifted complex is indicated by an asterisk (*). (B) Co-transfection assay of pCS3-$SIP1_{FS}$, pCS3-CZF3-Mut or pCS3-NZF3-Mut together with the p3TP-Lux reporter vector. The activity is expressed in percentage of full $SIP1_{FS}$ repressor activity, which is 100%.

Figure 10. **Ectopic activity of the mutated *Xbra2* promoter variants (Xbra2-Mut) in transgenic frog embryos**. (A) $SIP1_{FS}$ binding to the wild-type and mutated (Xbra-Mut; see Table I) *Xbra2* promoter elements. (B) Whole-mount *in situ* hybridisation for GFP mRNA of *Xenopus* embryos transgenic for a wild-type or point-mutated 2.1kb *Xbra2* promoter fragment driving a GFP reporter. All shown embryos were fixed at stage 11 and cleared for better visualisation of the signal. Percentages are indicative of intermediary phenotype (i.e., 35% of transgenic embryos displayed the normal *Xbra2* expression pattern and 65% showed ectopic expression).

Claims.

1. A method of identifying transcription factors such as activators and/or repressors comprising providing cells with a nucleic acid sequence at least comprising a sequence CACCT, preferably twice a CACCT sequence, as bait(s) for the screening of a library encoding potential transcription factors and performing a specificity test to isolate said factors.
2. A method of identifying transcription factors such as activators and/or repressors comprising providing cells with a nucleic acid sequence comprising one of the sequences CACCT-N-CACCT, CACCT-N-AGGTG, AGGTG-N-CACCT or AGGTG-N-AGGTG as bait wherein N is a spacer sequence of at least 8 base pairs.
3. A method according to claims 1 or 2 characterised in that the transcription factor comprises separated clusters of zinc fingers.
4. A method according to any of the preceeding claims wherein the sequence originates from a promoter region.
5. A method according to claim 4 wherein the promoter region is selected from Brachyury, α4-integrin, follistatin or E-cadherin.
6. Transcription factors obtainable by a method of any of the preceeding claims.
7. A method of identifying compounds with an interference capability towards transcription factors as defined in claim 6 by
   a) adding a sample comprising a potential compound to be identified to a test system composed of (i) an oligo nucleotide sequence comprising one of the sequences CACCT-N-CACCT, CACCT-N-AGGTG, AGGTG-N-CACCT or AGGTG-N-AGGTG as bait wherein N is a spacer sequence of at least 8 base pairs, (ii) a protein capable to bind said oligonucleotide sequence,
   b) incubating said sample in said system for a period sufficient to permit interaction of the compound or its derivative or counterpart thereof with said protein,
   c) comparing the amount and/or activity of the protein bound to the oligo nucleotide sequence before and after said adding and
   d) identification and optionally isolation and/or purification of the compound.

8. A method according to claim 7 wherein the protein is a Smad protein.
9. Test kit to perform the method of claim 7 comprising at least (i) an oligo nucleotide sequence comprising one of the sequences CACCT-N-CACCT, CACCT-N-AGGTG, AGGTG-N-CACCT or AGGTG-N-AGGTG wherein N is a spacer sequence of at least 8 base pairs and (ii) a protein capable to bind said oligonucleotide sequence.
10. Test kit to perform the method of claim 2 at least comprising a nucleic acid sequence comprising one of the sequences CACCT-N-CACCT, CACCT-N-AGGTG, AGGTG-N-CACCT or AGGTG-N-AGGTG wherein N is a spacer sequence of at least 8 base pairs.
11. A method for detecting an interaction between a first interacting protein and a second interacting protein comprising
    a) providing a suitable host cell with a first fusion protein comprising a first interacting protein fused to a DNA binding domain capable to bind a nucleic acid sequence comprising one of the sequences CACCT-N-CACCT, CACCT-N-AGGTG, AGGTG-N-CACCT or AGGTG-N-AGGTG wherein N is a spacer sequence of at least 8 base pairs,
    b) providing said suitable host cell with a second fusion protein comprising a second interacting protein fused to a DNA binding domain capable to bind a nucleic acid sequence comprising one of the sequences CACCT-N-CACCT, CACCT-N-AGGTG, AGGTG-N-CACCT or AGGTG-N-AGGTG wherein N is a spacer sequence of at least 8 base pairs,
    c) subjecting said host cell to conditions under which the first interacting protein and the second interacting protein are brought into close proximity and
    d) determining whether a detectable gene present in the host cell and located adjacent to said nucleic acid sequence has been expressed to a degree greater than expressed in the absence of the interaction between the first and the second interacting protein.

Abstract

The invention concerns a method of identifying transcription factors such as activators and/or repressors comprising providing cells with a nucleic acid sequence at least comprising a sequence CACCT as bait for the screening of a library encoding potential transcription factors and performing a specificity test to isolate said factors. Preferably the bait comprises twice the CACCT sequence, more particularly the bait comprises one of the sequences CACCT-N-CACCT, CACCT-N-AGGTG, AGGTG-N-CACCT or AGGTG-N-AGGTG wherein N is a spacer sequence of at least 8 base pairs.

The identified transcription factor(s) using the method according to the invention comprises separated clusters of zinc fingers such as for example a two-handed zinc finger transcription factor.

Figure 1

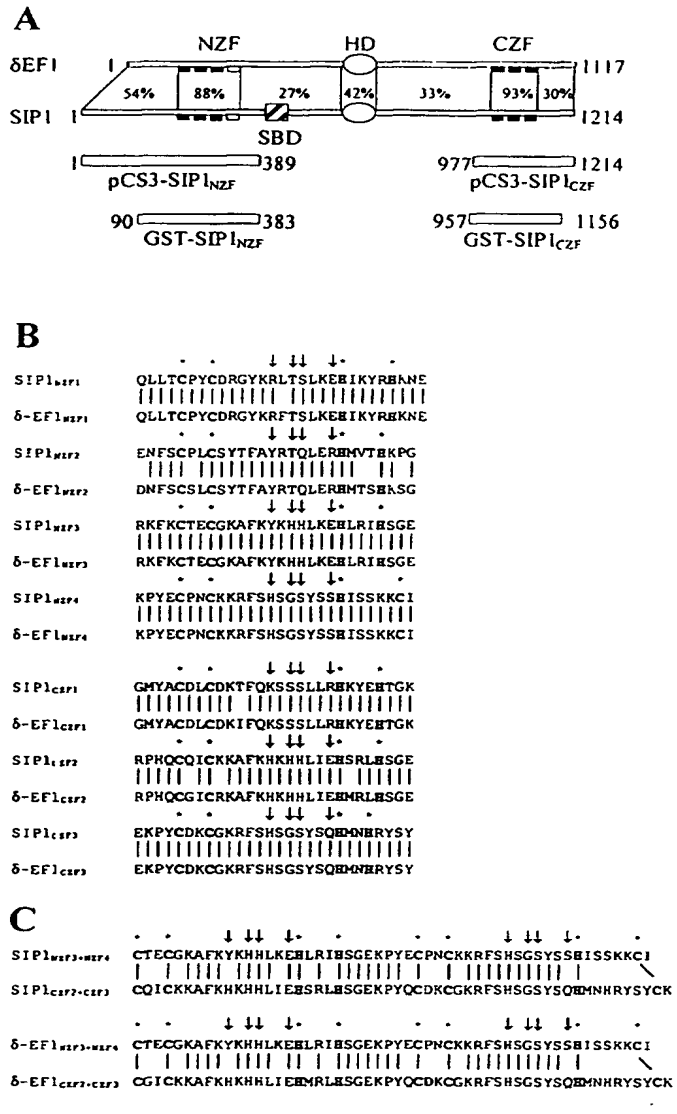


Figure 2

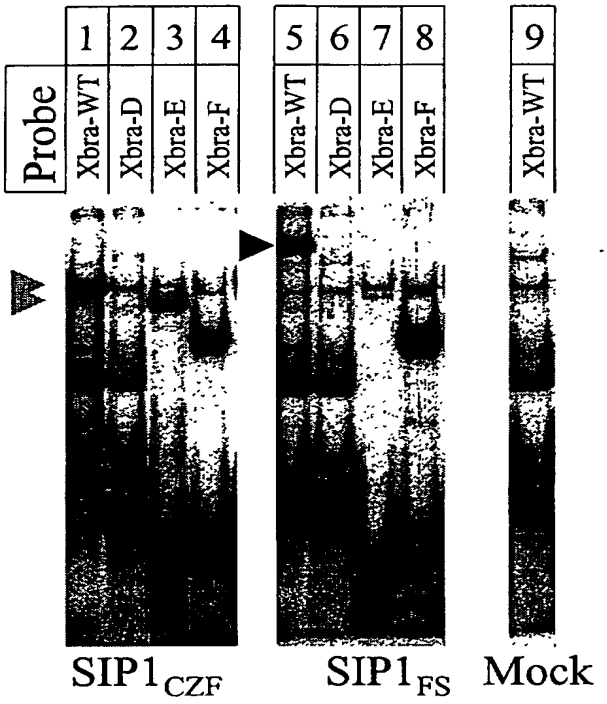
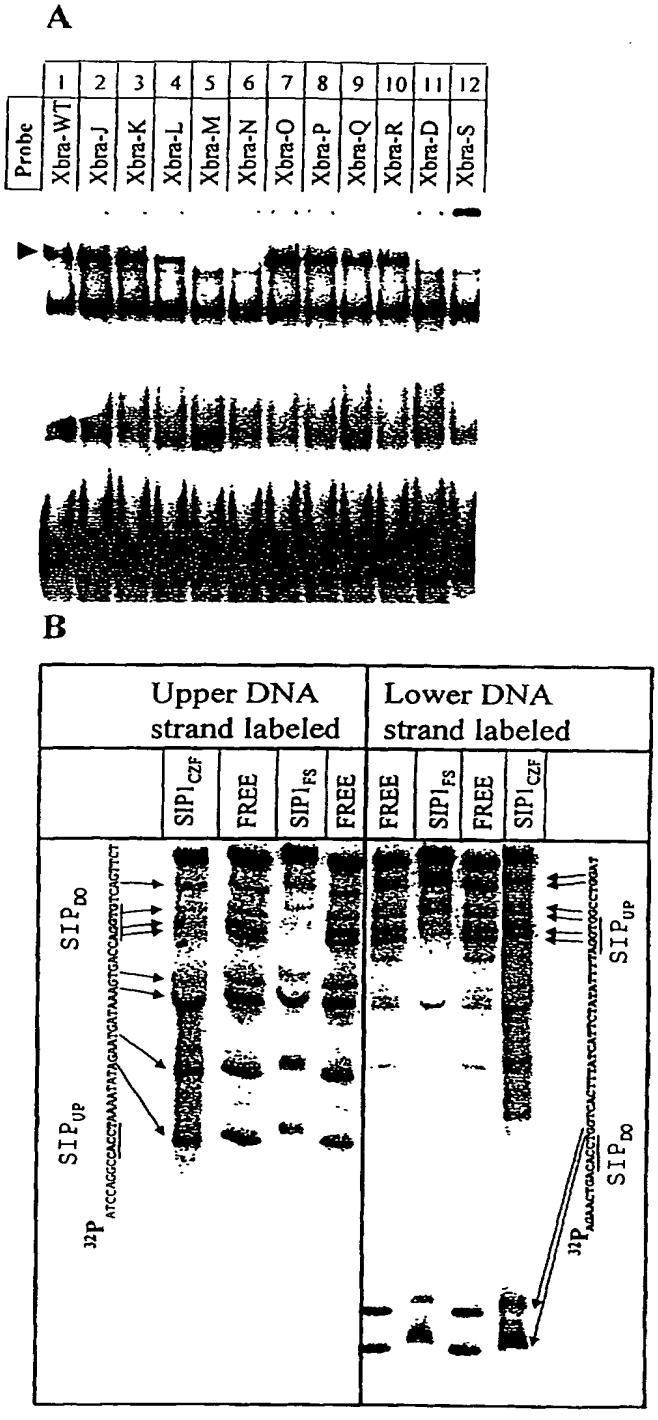


Figure 3



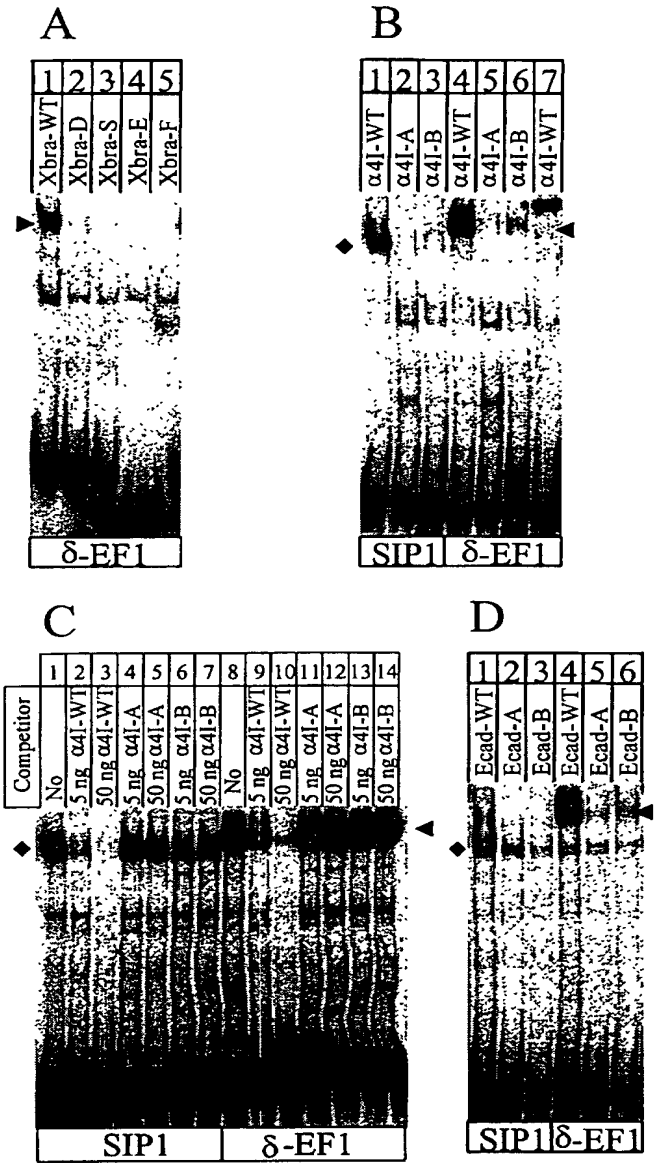


Figure 4

Figure 5

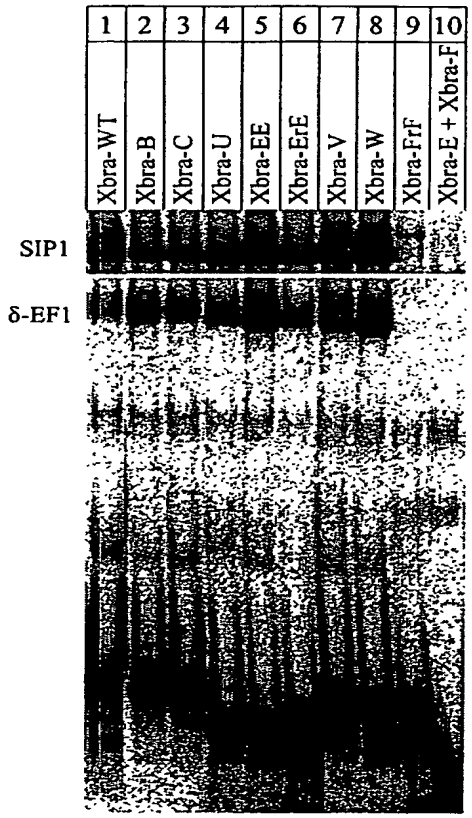


Figure 6

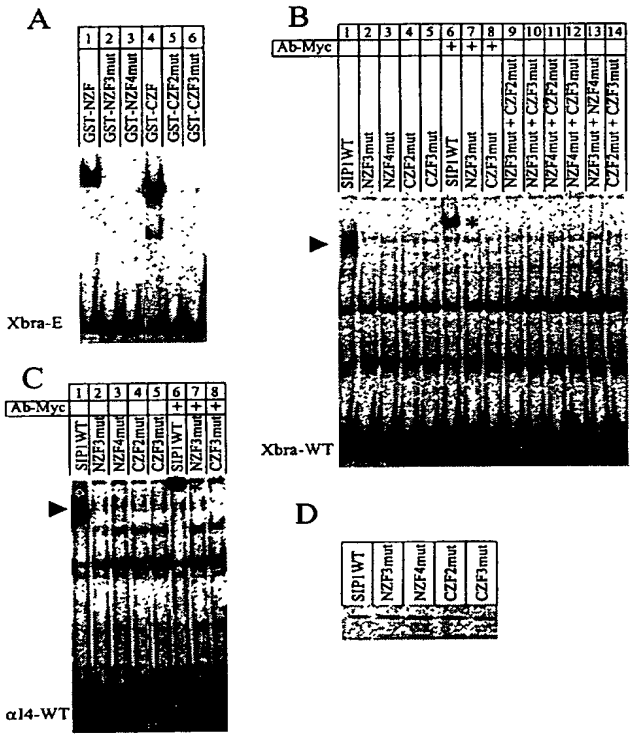


Figure 7

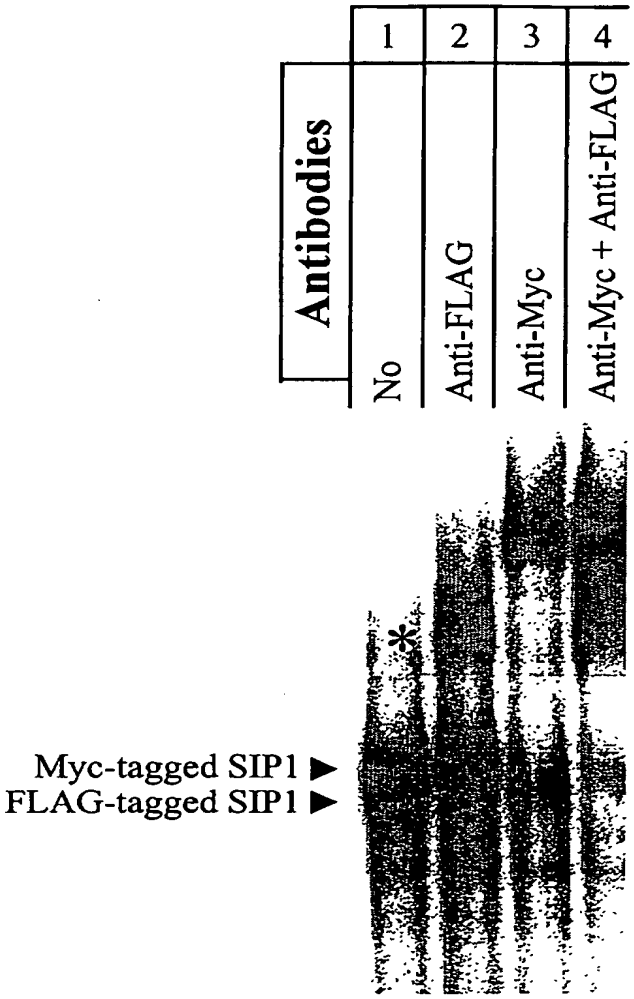


Figure 8

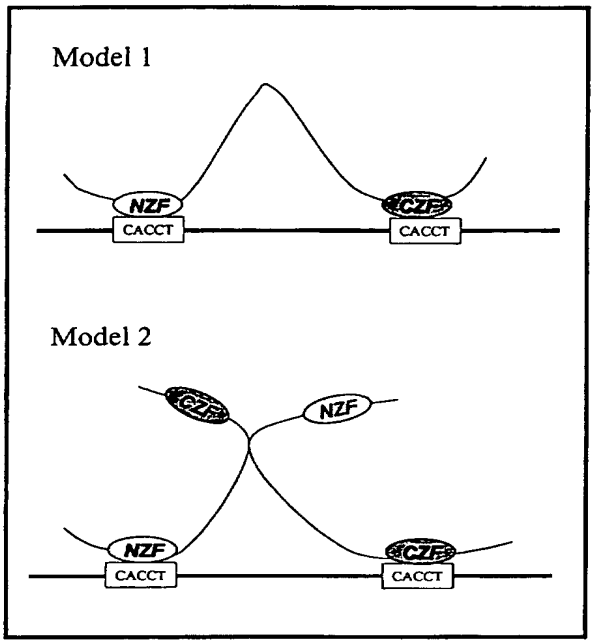

Model 1
NZF
CZF
CACCT
CACCT
Model 2
CZF
NZF
NZF
CZF
CACCT
CACCT

Figure 9

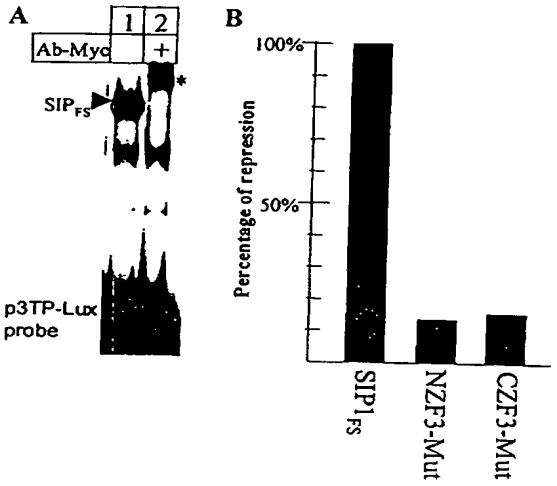
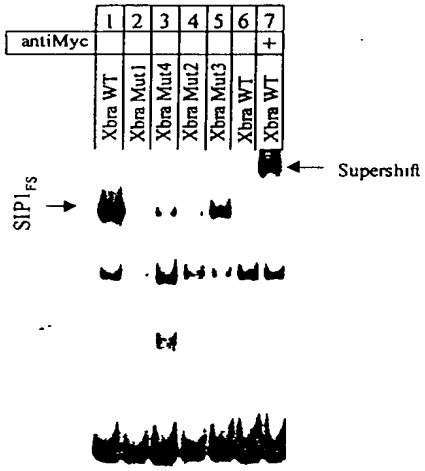


Figure 10

A



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